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Disease-specific motifs can be identified in circulating nucleic acids from live elk and cattle infected with transmissible spongiform encephalopathies

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ABSTRACT

To gain insight into the disease progression of transmissible spongiform encephalopathies (TSE), we searched for disease-specific patterns in circulating nucleic acids (CNA) in elk and cattle. In a 25-month time-course experiment, CNAs were isolated from blood samples of 24 elk (*Cervus elaphus*) orally challenged with chronic wasting disease (CWD) infectious material. In a separate experiment, blood-sample CNAs from 29 experimental cattle (*Bos taurus*) 40 months post-inoculation with clinical bovine spongiform encephalopathy (BSE) were analyzed according to the same protocol. Next-generation sequencing provided broad elucidation of sample CNAs: we detected infection-specific sequences as early as 11 months in elk (i.e. at least 3 months before the appearance of the first clinical signs) and we established CNA patterns related to BSE in cattle at least 4 months prior to clinical signs. In elk, a progression of CNA sequence patterns was found to precede and correlate with macro-observable disease progression, including delayed CWD progression in elk with PrP genotype LM. Some of the patterns identified contain transcription-factor-binding sites linked to endogenous retroviral integration. These patterns suggest that retroviruses may be connected to the manifestation of TSEs. Our results may become useful for the early diagnosis of TSE in live elk and cattle.

INTRODUCTION

Chronic wasting disease (CWD) was recognized as a naturally occurring transmissible spongiform encephalopathy (TSE) in elk and closely related species nearly 30 years ago (1). Although the causative agent of CWD is still the subject of debate (similar to other TSEs), neuro-invasion of orally challenged elk is thought to primarily occur via the vagosympathetic trunk (2), thus by-passing the blood–brain barrier. The definitive diagnosis of CWD currently involves immunohistochemistry to detect the occurrence of PrP^{CWD} protein in the brain, tonsils and lymph nodes, and histopathology to confirm the presence of spongiform lesions in the brain (3).

Recently, several publications indicate that CNAs might be used to obtain patterns related to diseases such as cancer and stroke (4). It is believed that serum CNAs are related to the lysis of cells in damaged tissues, necrosis and apoptosis (5). In humans, fetal CNAs have been shown to cross the maternal blood–brain barrier (6). It seems likely that CNAs from blood serum are linked to stress-related activities throughout the body, including

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neuronal death associated with TSEs. In the case of TSE, an additional source of CNAs may be exosome-like structures that are expelled by infected cells, as reported for scrapie (7,8). Given the potential benefits of a live blood test, an elk CNA study was undertaken to determine if a correlation exists between CWD and specific CNA pattern distributions in healthy versus diseased animals. In addition, blood CNAs from cattle, which were perorally challenged with BSE and had progressed to approximately 4 months before clinical signs, were investigated by comparison to sham-inoculated animals.

MATERIALS AND METHODS

Elk study

Twenty-four weaned *Cervus elaphus* females were obtained from two commercial farms in Alberta (Canada). Both farms had no previous cases of CWD, no history of importing animals from herds with CWD, and had been actively participating in CWD surveillance programs. The animals were genotyped and separated into groups, such that both MM and LM animals were distributed over the study period. The rare LL genotype (9) was not present in this study, most likely due to the relatively small number of animals used. The LM genotype is known to be linked with a slower CWD disease progression as compared to the MM genotype (10). The elk were perorally challenged with 10 ml of a 10% brain homogenate (tested for CWD by western blot) and 90% saline (w/v). CWD-positive brain homogenate was obtained from pooled elk tissue, kindly provided by Dr A. Balachandran (Canadian Food Inspection Agency, Fallowfield) and fed to 19 animals. The CWD-negative homogenate was obtained from an elk that died of unrelated causes and tested negative for CWD and fed to five animals. Blood was sampled in a monthly schedule and stored at -20°C. The animals were euthanized according to an experimental schedule and/or for humane reasons (Table S1).

Cattle study

Serum samples were obtained from 29 Fleckvieh cattle (Simmental breed) 40 months post inoculation, which had been fed 100 g of either PrP^{res}-positive brain stem macerate (16 animals) or non-infected brain material (13 animals), respectively (11).

The CNA extraction, DNA sequencing and bioinformatics analysis followed the same protocol in the elk and bovine studies.

Preparation of sera

Serum was obtained and prepared as described elsewhere (12). Frozen serum was thawed at 4°C and cell debris was removed by brief centrifugation at $4000 \times g$ for 20 min. The supernatant was subjected to 35 min centrifugation at $20\,000 \times g$ and the pellet was used for further analyses.

Nucleic acid extraction, general and CNA-specific amplifications

The WGA4 GenomePlex® Single Cell Whole Genome Amplification Kit (Sigma) was used for the nucleic-acid extraction and amplification of total nucleic acids according to the manufacturer's protocol. The amplified DNA was purified and 5 ng DNA of each sample was used for the circulating nucleic-acid-specific amplification using quasi-random primers with a universal 5′ adapter using a proof-reading PCR system.

Disease-specific CNA subtraction

PCR products for each animal after oral infection were hybridized with a 10-fold overshoot of biotinylated pre-infection PCR amplicons for 70 h. The latter amplicons contained dUTP for subsequent digestion of unbound products. After binding to strepavidin-coated magnetic beads, followed by magnetic separation, the unbound fraction was collected and subjected to digestion with uracil DNA *N*-glycosylase to avoid a carry-over of unbound pre-infection amplicons. The remaining undigested PCR-products were used for further processing and sequencing. All pre-infection samples (controls) were used directly for sequencing without any selection.

DNA sequencing

The PCR products were sequenced directly using a Roche/454 genome sequencer (GS-FLx) according to the manufacturer's instructions (conducted at Eurofins-Medigenomix, Martiensried, Germany). The raw sequences were trimmed to eliminate adapter/primer sequences and identification tags.

Bioinformatics

Figure 1 provides an overview of the bioinformatics analysis performed. An all-versus-all search of the elk dataset using four TimeLogic DeCypher boards (http://www.timelogic.com) all patterns occurring for a minimum of $20\times$ (based on the number of infected animals), with none of the top 100 BLAST (13) matches occurring in non-target samples, yielded 10 634 motifs specific to control and infected animals up to 5 months after infection; and 4933 motifs for animals at least 8 months after infection (motifs of 35 bases or less, using a sliding window for longer matches). Each of these motifs was searched against the entire elk CNA dataset using a simulated thermodynamic annealing approach (14). The motifs were further filtered to include only those readily and unambiguously detectable using PCR: i.e. those matching without any critical off-target binding within 4°C under PCR conditions of 10^{-6} M DNA and 0.15 M Na^{+}. In the two-timepoint bovine dataset, 3261 control-only motifs and 2896 motifs present in infected-only cows were found with the same approach. In addition, each CNA sequence for elk and cattle was searched against the NCBI expressed sequence tag (EST) and non-redundant protein databanks using BLAST to identify potential functions based on sequence similarity.

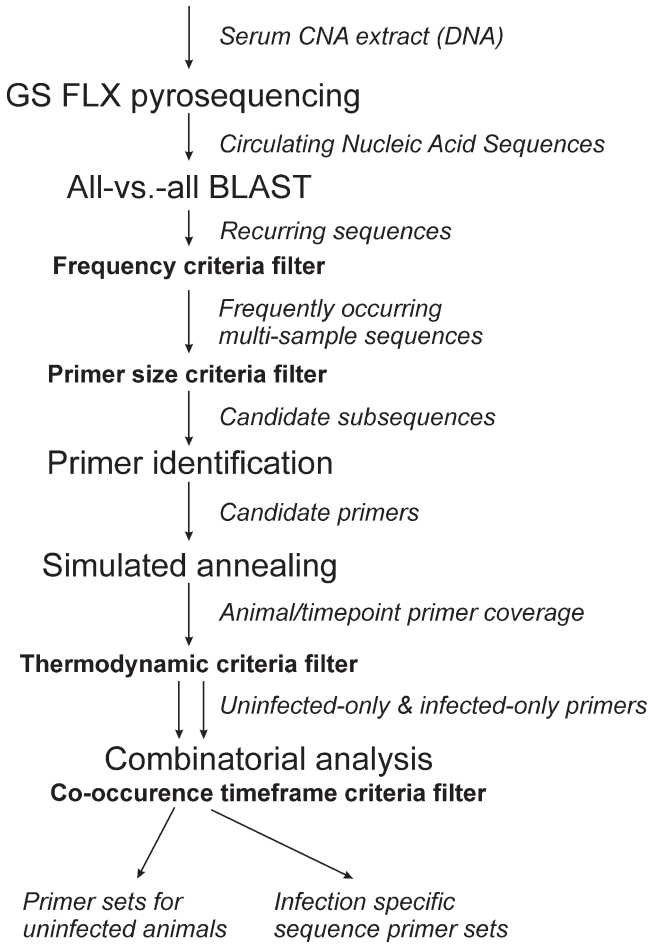


Figure 1. Bioinformatics workflow to design disease-indicator primers for circulating nucleic acids. The list of candidate indicator sequence segments is consecutively filtered based on desired criteria. An initial BLAST search's results are refined for practicality using frequency, sample and length criteria, then a simulated annealing of the candidate primers to the entire dataset is performed to avoid off-target matches. Finally, the candidate primers are placed into infected and uninfected target groups, and co-occurrence criteria (e.g. found in same month's sample from all infected animals) are gradually relaxed (e.g. within a 3-month span in all infected animals) until 100% specificity and 100% selectivity are achieved for a set of primers.

RESULTS

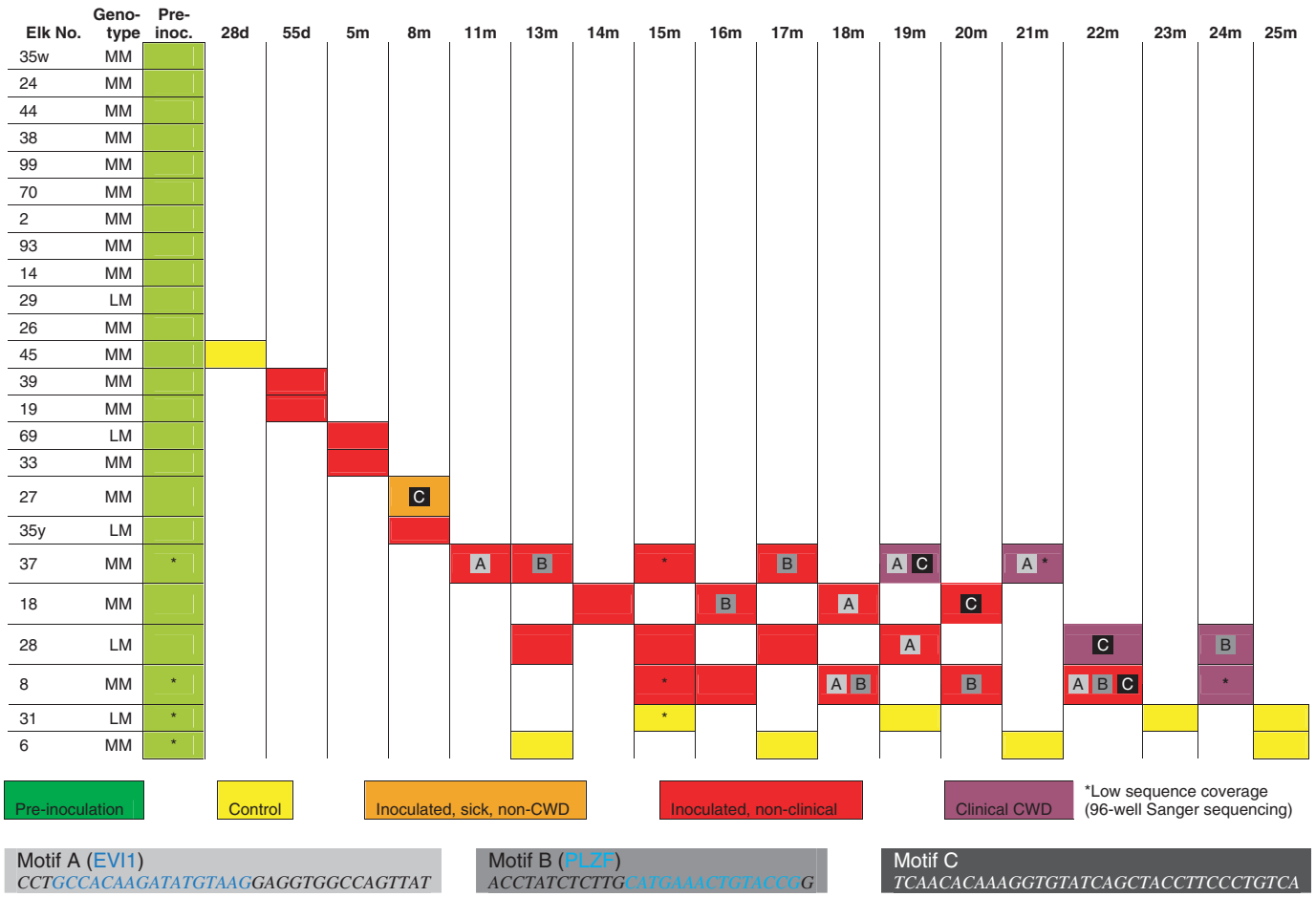
A total of 657 431 quality elk sequences were elucidated, including 401 733 from animals post-infection, with an average read length of 163 bases. Only 2% of the CNAs showed strong sequence similarities (BLAST expected-value $<10^{-25}$), when searched against the public protein and EST datasets, while 10% showed intermediate (BLAST expected-value $<10^{-15}$) similarities. Most of the matches were against ESTs from the public databanks with unknown function, with no recurring matches to protein functions other than reverse transcriptase (approximately 1.3% of the CNAs).

In elk, three types of motifs which are representative of different CWD stages could be deduced (see Table 1 for all CNA motifs described here and Supplementary Table S1 for more detail). While we were not able to identify any universal control-only motif(s), that were characteristic exclusively to non-infected elk, we were able to identify a 'first stage' motif set, covering animals before clinical signs, up to 5 months post infection. A combination of four individual motifs is needed to capture all of the 24 prebleed (before inoculation) and 28-day/55-day/5-month (post-inoculation) samples. No significant function could be attributed to any of these motifs. Two motifs, which occur only in the near-clinical elk samples, were discovered, hereafter called motifs A and B. The first appearances of motifs A and B in the animals with the MM genotype were at 11 and 13 months; and animals with the LM genotype at 19 and 24 months after inoculation, respectively. Sera sampling and motif occurrence details can be found in Supplementary Table S2. Motif A was found to contain a match to an ecotropic viral integration 1 (EVI1) protein binding site, with 100% core similarity and 95% overall matrix similarity, when searched using the MatInspector database (15). Based on a random sample of 6000 CNAs, we estimate an *e*-value of 0.01 for any of MatInspector's EVl1 patterns in our CNA dataset. Motif B contains a promyelotic leukemia zinc finger (PLZF)-binding site, with 100% core similarity, a 92% overall matrix similarity, and an *e*-value of ~0.005. In general, the timing of the appearance motifs A and B is dependent on the animals' genotype and overall health, preceding clinical signs by at least 3 months. A third motif type, motif C, occurs at the terminal stage in all infected animals. This motif does not contain any MatInspector hits as strong as the EVI1 and PLZF indicators. The 'terminal stage' motif was also to present at month 8 in a sample of one infected animal, which was sacrificed due to an extensive area of inflammation in the cheek (most likely caused by a skin wound). Oral wounds may greatly accelerate prion neuroinvasion (16), therefore in this case further experimentation is required to determine if this motif is specific to CWD infected-only animals or terminal cases in general.

A total of 595 037 quality bovine sequences were elucidated, including 311 786 from animals post-infection, with an average read length of 188 bases. In this dataset, four motifs are sufficient to cover all control animals (Table 2). Similar to the elk sample, no significant function could be attributed to any of these motifs (Supplementary Table S2). A search for shared BSE-only motifs demonstrated a high bias towards only one quarter of the 16 animals, making a universal pattern set for BSE harder to obtain. A selection of five motifs is sufficient to detect any of the 16 BSE animals at 40 month, but none of the controls. None of these five BSE-only motifs had statistically significant matches to any known transcription factors. Based on our time series data from elk, the lack of universal sequences in our cattle dataset is not surprising, as indicator motifs tend to appear in the sequence data in low frequency and in a progressive series that is dependent on the health of the individual animals.

In general, reverse transcriptases (*pol*) genes were well represented in the cattle CNA sequences (Table 3). Several infected animals possessed matches to an uninterrupted *pol* coding region (Table 3, panel a), while several non-infected animal sequences matched another uninterrupted *pol* coding region (Table 3, panel b).

Table 1. Progressive appearance of CNA motifs exclusive to CWD-infected elk, at least 3 months before clinical signs, and delayed in the CWD-resistant genotype (LM)



Motif A contains an EVI1 protein binding site. Motif B contains a PLZF binding site. EVI1 is a known promoter for PLZF. Motif C, which appears at the terminal stage in infected animals, has no known function.

Table 2. Recurring sequence motifs in BSE-infected and non-infected cattle

Sequence Motif
Non-infected animals
GGTGGGTTGCCTGACACCCTGG
CTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTC
GGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGC
TGGGTATGGATAGGACTGCTAGGAATACGTGTTGG
Infected animals
AATCCTAGCTTTCTCCATTGAGGACCG
AAATTCTCGAGCATCTCCCCCCAAGAAAAACCAG
CTGCCAATGCAGGAGACGTGGG
AAGAGACCTGGGTTCGATCCCTAGG
TGCAACATGCCAGGCTCCCCT

The motifs were discovered using the same technique as used for the elk in Table 1. Serum samples were obtained from 29 Fleckvieh cattle (Simmental breed) 40 months post-inoculation (i.e. at least 4 months before clinical signs).

Additional retroviral-like sequences with in-frame stop codons are found regardless of infection state (Table 3 panel c). Sequences (a) and (b) from Table 3 may point towards the detectability of BSE-discriminating retroviral patterns, but this will need to be confirmed with a time series study. Using the Smith–Waterman algorithm (17), the reverse transcriptase sequences identified in our samples were found to be more closely related at the protein level to various murine leukemia viruses (*e*-value 10^{-34} for the most frequently occurring *pol*) than to known bovine leukemia viruses (*e*-value 10^{-12}).

DISCUSSION

EVI1 is a common site for retroviral integration in mammalian genomes, where viral integration can promote protein expression of EVI1 (18). The EVI1 protein has been shown to act as a transcriptional promoter for a small number of genes, protecting the binding sites

Table 3. Recurring endogenous retrovirus-like sequences in bovine CNAs

	No. of non-infected animals (no. of total sequence occurrences)	No. of infected animals (no. of total sequence occurrences)	Best homology	Best murine homology	CNA contains stop codon in-frame	Present in bovine genome (>95% DNA ID)
(a)	0 (0)	5 (12)	10^{-47} gag/pol protein *(Populus trichocarpa)*	10^{-14} putative gag-pro-pol polyprotein *(xenotropic murine leukemia virus)*	No	No
(b)	5 (5)	0 (0)	10^{-18} predicted pol polyprotein from transposon 297, partial *(Canis familiaris)*	10^{-11} pol protein *(Recombinant M-MuLV/RaLV retrovirus)*	No	Truncated match
	5 (5)	3 (3)	10^{-29} predicted protein *(Homo sapiens)*	10^{-23} polymerase *(murine leukemia virus)*	No	Truncated match
	2 (2)	5 (4)	10^{-37} gag protein *(Bos taurus)*	10^{-10} gag polyprotein *(xenotropic murine leukemia virus)*	No	Yes
(c)	2 (4)	4 (5)	10^{-23} pol protein *(human endogenous retrovirus HCML-ARV)*	10^{-19} gag-pol polyprotein *(murine leukemia virus)*	Yes	Truncated match
	5 (7)	8 (10)	10^{-49} polyprotein *(multiple sclerosis associated retrovirus)*	10^{-34} pol polypeptide *(murine leukemia virus)*	Yes	Yes
	2 (2)	3 (3)	10^{-8} pol protein *(human endogenous retrovirus HCML-ARV)*	10^{-6} pol polypeptide *(murine leukemia virus)*	Yes	Yes

(a) One *pol*-like sequence was found exclusively in (5 of 16) infected animals, but may not be endogenous to cattle as it is not found in the current version of the draft bovine genome (13 September 2007). (b) One *pol*-like sequence was found exclusively in non-infected animals (5 of 13). (c) One *gag*-like and four *pol*-like sequences are recurrent in both PrP^{res}-infected and non-infected populations, indicating that some endogenous retrovirus sequences are consistently detectable regardless of prion exposure.

from DNAse I digestion (19). The transcription factor's protective effect (20) may explain the occurrence of motif A in the blood CNAs, especially if EVI1 is being expressed beyond what is normally required for its limited functions in adults (21). One of the genes promoted by EVI1 is the transcription factor PLZF, whose target is found in motif B, thus the function of motifs A and B can be directly linked.

EVI1 expression has thus far not been linked to TSE processes in the literature, but single-stranded RNA retroviruses that preferentially integrate into EVI1 have been. Endogenous murine leukemia virus, which is known to re-integrate into EVI1, has been shown *in vivo* to replicate upon introduction of scrapie (22). Conversely, Moloney murine leukemia virus infection has been shown *in vitro* to strongly enhance scrapie infectivity (23). Instances of Moloney-like DNA sequences are also found throughout the latest version of the bovine genome assembly (13 September 2007), with 26 genome regions having a BLAST *e*-value $<10^{-100}$. Retroviruses may therefore act as cofactors for TSE pathogenesis (24,25). Retrovirus-induced activation of EVI1 during the course of CWD infection could provide a biological basis for the pair of motifs that we detected in elk. These results are also well aligned with our previous findings of human endogenous retroviral associations with progressive neurologic diseases (26).

Growing evidence [e.g. (27,28)] shows that PrP^{res} alone has dramatically lower infectivity than TSE agent particles. A link between general retroviral activity and TSEs was first proposed almost twenty years ago (29). While such a link is speculative, two types of data specifically support an endogenous retroviral cofactor theory. First, the prion agent is released into cell culture in association with exosome-like structures and viral particles of endogenous origin (8). Second, a growing body of literature suggests an antiretroviral role for the PrP protein (30–32). More specifically, recent results (32) show that PrP is highly up-regulated in response to increased activity of murine endogenous retroviruses. The mRNA level of PrP also increases after scrapie agent inoculation in lymphoid tissue (33). If PrP^{cwd}-prompted lymphoid retroviral activity is responsible for EVI1 detection in CNAs, this could explain its early detectability: lymphoid tissues accumulate PrP^{cwd} before the central nervous system (34).

The nucleic-acid-binding properties of PrP (35), and the presence of PrP^{cwd} in cervid blood (36) mean that alternative explanations for the presence of the motifs are possible. Retroviral RNA may play a role in the transformation of PrP^{c} to PrP^{d} (37), or may conversely reduce PrP^{c} availability (38). While an aptamer role is possible, a Smith–Waterman search of both the motifs and common C-type retroviruses against 87 prion-targeting nucleic-acid aptamers found in the literature and patents does not yield any matches. The possibility remains that the motif sequences may bind PrP in a non-sequence-specific manner (39). A simple present/absent criteria was used for unbiased motif discovery in a randomly sequenced CNA dataset: these motifs will inform our targeted characterization and quantification of disease-specific CNAs and any associated complexes going forward.

The biological mechanism by which the CNAs originate still remains uncertain, but our data support the idea that it is possible to detect host reaction to the CWD and BSE

infectious agents via CNAs in live animals, and importantly, before clinical signs appear. Further studies will help to validate the specificity of patterns for TSEs versus brain trauma and other neurological diseases, as well as the consistency of the patterns in naturally occurring CWD and BSE cases. The information from the elk study demonstrates that a time course analysis of the blood CNAs can greatly improve our ability to determine the diagnostic signals present in slowly developing, poorly understood diseases such as TSEs. We consider that the patterns described here provide a starting point for the development of a relatively simple, cost-effective live animal test. For instance, in the future PCR-based tests, pooled samples could be used on a large scale to eliminate infected animals from the human food chain, even before the onset of clinical signs. In this context, it is also interesting that we were able to identify a combination of patterns in elk, which was only found in control animals and animals up to 5 months after infection, and patterns in cattle that were only present in the controls (when compared to animals 40 months after infection). Adding these patterns to the eventual screening strategy could certainly enhance the accuracy of a live animal testing system.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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